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## **Recovery of donor hearts after circulatory death with normothermic extracorporeal machine perfusion**

Tolboom, Herman ; Makhro, Asya ; Rosser, Barbara A ; Wilhelm, Markus J ; Bogdanova, Anna ; Falk, Volkmar

**Abstract:** **OBJECTIVES** A severe donor organ shortage leads to the death of a substantial number of patients who are listed for transplantation. The use of hearts from donors after circulatory death could significantly expand the donor organ pool, but due to concerns about their viability, these are currently not used for transplantation. We propose short-term ex vivo normothermic machine perfusion (MP) to improve the viability of these ischaemic donor hearts. **METHODS** Hearts from male Lewis rats were subjected to 25 min of global in situ warm ischaemia (WI) (37°C), explanted, reconditioned for 60 min with normothermic (37°C) MP with diluted autologous blood and then stored for 4 h at 0-4°C in Custodiol cold preservation solution. Fresh and ischaemic hearts stored for 4 h in Custodiol were used as controls. Graft function was assessed in a blood-perfused Langendorff circuit. **RESULTS** During reconditioning, both the electrical activity and contractility of the ischaemic hearts recovered rapidly. Throughout the Langendorff reperfusion, the reconditioned ischaemic hearts had a higher average heart rate and better contractility compared with untreated ischaemic controls. Moreover, the reconditioned ischaemic hearts had higher tissue adenosine triphosphate levels and a trend towards improved tissue redox state. Perfusate levels of troponin T, creatine kinase and lactate dehydrogenase were not significantly lower than those of untreated ischaemic controls. The micro- and macroscopic appearance of the reconditioned ischaemic hearts were improved compared with ischaemic controls, but in both groups myocardial damage and oedema were evident. **CONCLUSIONS** Our results indicate that functional recovery from global WI is possible during short-term ex vivo reperfusion, allowing subsequent cold storage without compromising organ viability. We expect that once refined and validated, this approach may enable safe transplantation of hearts obtained from donation after circulatory death.

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# Recovery of donor hearts after circulatory death with normothermic extracorporeal machine perfusion<sup>†</sup>

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## Abstract

**OBJECTIVES:** A severe donor organ shortage leads to the death of a substantial number of patients who are listed for transplantation. The use of hearts from donors after circulatory death could significantly expand the donor organ pool, but due to concerns about their viability, these are currently not used for transplantation. We propose short-term *ex vivo* normothermic machine perfusion (MP) to improve the viability of these ischaemic donor hearts.

**METHODS:** Hearts from male Lewis rats were subjected to 25 min of global *in situ* warm ischaemia (WI) (37°C), explanted, reconditioned for 60 min with normothermic (37°C) MP with diluted autologous blood and then stored for 4 h at 0–4°C in Custodiol cold preservation solution. Fresh and ischaemic hearts stored for 4 h in Custodiol were used as controls. Graft function was assessed in a blood-perfused Langendorff circuit.

**RESULTS:** During reconditioning, both the electrical activity and contractility of the ischaemic hearts recovered rapidly. Throughout the Langendorff reperfusion, the reconditioned ischaemic hearts had a higher average heart rate and better contractility compared with untreated ischaemic controls. Moreover, the reconditioned ischaemic hearts had higher tissue adenosine triphosphate levels and a trend towards improved tissue redox state. Perfusate levels of troponin T, creatine kinase and lactate dehydrogenase were not significantly lower than those of untreated ischaemic controls. The micro- and macroscopic appearance of the reconditioned ischaemic hearts were improved compared with ischaemic controls, but in both groups myocardial damage and oedema were evident.

**CONCLUSIONS:** Our results indicate that functional recovery from global WI is possible during short-term *ex vivo* reperfusion, allowing subsequent cold storage without compromising organ viability. We expect that once refined and validated, this approach may enable safe transplantation of hearts obtained from donation after circulatory death.

**Keywords:** Cardiac transplantation • Donation after circulatory death • Organ preservation • Ischaemia–reperfusion • Machine perfusion

## INTRODUCTION

Cardiac transplantation is the only curative therapy for end-stage heart failure, but is limited by a severe shortage of suitable donor organs. Because of this, it has been estimated that ~17% of all patients listed for cardiac transplantation die before a suitable donor heart becomes available [1, 2]. Extending graft criteria to include organs from donation after circulatory death (DCD) could expand the donor organ pool significantly [1].

Although DCD is gaining momentum in solid organ and lung transplantation [3–5], hearts from DCD donors are generally not

used for transplantation [6, 7]. Consequently, donor hearts are almost exclusively obtained from brain-dead, heart-beating donors. During heart-beating donor organ procurement, the heart is flushed with ice-cold cardioplegic solution directly after aortic clamping, causing instant cardiac arrest and rapid cooling, dramatically lowering the heart's metabolic rate. However, because the heart's metabolism does not drop to zero during the time its oxygen supply is cut-off, intracellular energy stores, in particular adenosine triphosphate (ATP), are gradually exhausted. Nevertheless, when the donor hearts is kept immersed in preservation solution at 0–4°C, safe cold storage (CS) up to 4–5 h is possible [8]. In a typical DCD setting, cardiac arrest is the result of anoxia upon withdrawal from a ventilator. Because initially, the

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heart continues to beat but is devoid of oxygen, myocardial metabolism switches from oxidative to anaerobic, leading to rapid loss of intracellular ATP, acidosis and accumulation of metabolites, thereby compromising graft viability [9, 10]. DCD hearts have been transplanted on a very limited scale [11], but due to concerns about the combined impact of DCD and the cold ischaemia inherent to CS, they are generally not used for transplantation.

Normothermic machine perfusion (NMP) has been explored as alternative to CS in order to improve the function of DCD hearts. During NMP, the heart is kept in a beating state by continuous perfusion with warm, oxygenated perfusate [9, 12]. Experimentally, continuous NMP has enabled recovery from ischaemia and has improved the viability of hearts from DCD [7, 11, 13, 14]. However, compared with CS, NMP is far more challenging to implement, especially during transportation of the donor organ. We have, therefore, investigated whether an initial period of NMP, during which the graft can recover from ischaemia, may be combined with a clinically relevant period of CS, enabling transportation in an ice chest as usual.

## METHODS

All experiments were in compliance with the Swiss and cantonal legislation regarding humane care of animals. Isoflurane (5% for induction and 2% for maintenance of anaesthesia; Abbott, Baar, Switzerland) in a 1:1 O<sub>2</sub>/N<sub>2</sub>O mixture (PanGas, Dagmarsellen, Switzerland) was used as anaesthetic for all experiments.

### Experimental design

Male Lewis rats (Charles River, Sulzfeld, Germany) weighing 275–325 g ( $n = 6$  per group) were placed on a heated operating table. Heparin (1000 IU/kg) (B Braun, Sempach, Switzerland) was given intravenously, the abdomen was opened with a transverse incision and 4 ml of blood was collected in syringe containing 500 IU of heparin. The hearts in the fresh control group were then excised, flushed with Custodiol and stored on ice while immersed in Custodiol for 4 h. The hearts in both ischaemic groups were subjected to 25 min of warm (37°C) ischaemia after inducing asphyxiation by dissection of the diaphragm and allocated to the respective preservation protocols (Fig. 1). Following CS, graft function was assessed in a blood-perfused Langendorff circuit. Additionally, hearts from each group ( $n = 5$  per group) were snap-frozen in liquid nitrogen before and after CS for determination of myocardial ATP and reduced (GSH) and oxidized (GSSG) glutathione.

### Cold storage

Excised fresh and ischaemic hearts were placed in ice-cold saline, an 18-G cannula (Terumo, Spreitenbach, Switzerland) was positioned in the aorta, secured with a 4-0 silk ligature and the heart was slowly flushed with 10 ml of ice-cold preservation solution (Custodiol; Dr. F. Köhler Chemie, Bensheim, Germany). The hearts were kept immersed in preservation solution on ice for 240 min.

### Perfusion circuit

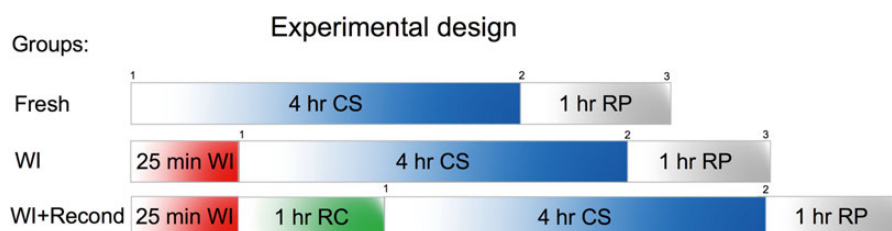
The perfusion circuit consisted of a jacketed perfusion chamber, an oxygenator and a heat exchanger, all designed and fabricated specifically for this purpose, as well as a peristaltic pump. The oxygenator with a priming volume of 1 ml was constructed with Oxyphan hollow fibres (Membrana, Wupperthal, Germany) and gassed with a humidified gas mixture containing 95% O<sub>2</sub>/5% CO<sub>2</sub> (PanGas). The total priming volume of the perfusion circuit was ~8 ml.

### Ex vivo reconditioning

Following warm ischaemia (WI), the hearts in the reconditioning (RC) group were weighed and placed in the perfusion circuit primed with autologous blood diluted 1:1 with Krebs–Henseleit buffer containing 10 mmol/l glucose, 117 NaCl, 5.9 KCl, 25 NaHCO<sub>3</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 1 CaCl<sub>2</sub>, 0.512 MgCl<sub>2</sub> (all chemicals were purchased from Sigma-Aldrich Chemie, Buchs, Switzerland) and 500 IU of heparin. Retrograde NMP (37°C) took place at a constant flow rate of 2.5 ml/g/min for 60 min (see Table 1 for the operating parameters). ECG (aVL projection) was recorded continuously. At the end of the RC phase, hearts were removed from the perfusion circuit, flushed with 10 ml of preservation solution and kept immersed in preservation solution at 0–4°C for 240 min.

### Langendorff reperfusion

At the end of the respective preservation intervals, the hearts were placed in the perfusion circuit, a balloon-tipped catheter (Hugo Sachs Elektronik/Harvard Apparatus, March-Hugstetten, Germany) was inserted into the left ventricle through an incision in the left atrium and the hearts were perfused in a retrograde manner for 60 min at 37°C with autologous blood diluted 1:1 with Krebs–Henseleit buffer at a constant flow rate of 2.5 ml/g/min (Table 1). After an equilibration period of 10 min, the developed pressure, heart rate and ECG were continuously recorded with signal amplifiers connected to an analogue-digital transducer



**Figure 1:** <sup>1</sup>Tissue samples taken from all groups prior to cold storage, perfusate samples taken from the WI + Recond group, <sup>2</sup>tissue samples taken from all groups following cold storage, and <sup>3</sup>tissue and perfusate samples taken from all groups following Langendorff reperfusion. WI: warm ischaemia; CS: cold storage; RP: reperfusion; RC: reconditioning.

**Table 1:** Operating parameters during reconditioning and Langendorff reperfusion

Temperature (°C)	37
Flow rate (ml/g/min)	2.5
pO <sub>2</sub> (mmHg)	150–250
pCO <sub>2</sub> (mmHg)	35–45
pH	7.3–7.4
Haemoglobin (g/dl)	6–7

(Power Lab; ADInstruments, Oxfordshire, UK). Perfusate pO<sub>2</sub>, pCO<sub>2</sub>, haematocrit and pH were monitored using a Stat Profile pOX Plus blood analyser (Nova Biomedical, Waltham, USA). Following reperfusion, the hearts were immersed in ice-cold saline and weighed. The hearts were then divided into four 2–3 mm thick transverse sections, which were snap-frozen in isopentane/liquid nitrogen, or fixed in 4% formaldehyde. Perfusate samples were centrifuged (16 000 rpm) at 4°C for 2 min, and the supernatant was pipetted off and stored at –20°C.

## Analysis

**Graft viability.** Myocardial injury was assessed by measuring the perfusate levels of cardiac troponin T using an electrochemiluminescent immunoassay and lactate dehydrogenase and creatine kinase (CK) using an enzymatic UV assay, and K<sup>+</sup> was measured using an ion-selective electrode (all from Roche Diagnostics, Rotkreuz, Switzerland).

**Oxidative stress.** The ratio of reduced/oxidized glutathione (GSH/GSSG) was used to assess tissue redox state as an indicator of oxidative stress. Tissue samples were homogenized in equal volumes of solutions containing either 100 mM KCl and 10 mM MOPS-KOH or 5% trichloroacetic acid in distilled water. After centrifugation, the supernatant was processed as described in detail elsewhere [15]. The half-cell redox potential (E<sub>hc</sub>) for the GSH:GSSG couple was then calculated using the following equation:

$$E_{hc} = -240 - \frac{59.55}{2} \log \frac{[GSH]^2}{[GSSG]}$$

where [GSH] and [GSSG] stand for the intracellular GSH and GSSG content per g wet tissue.

**Tissue energy state.** Tissue ATP levels of homogenized myocardial tissue were measured using a bioluminescence kit (Sigma-Aldrich Chemie). Analysis was performed using a Sirius luminometer (Berthold Detection Systems, Pforzheim, Germany).

**Histology.** Tissue slices were fixed in 4% formalin, embedded in paraffin, cut to 4-μm-thick sections and stained with haematoxylin-eosin. Cell death was evaluated through terminal deoxynucleotide transferase-mediated dUTP nick-end labelling on 6-μm-thick formalin-fixed paraffin embedded sections using a CardioTACS *in situ* apoptosis detection kit (R&D Systems, Abingdon, UK).

## Statistical analysis

The mean and maximum heart rate, mean contractility index and dP/dT of the respective hearts were calculated using the Lab Chart software (ADInstruments). Comparison between the experimental groups was performed with the GraphPad Instat.V3.05 software (GraphPad Software, Inc., La Jolla, USA), using analysis of variance (ANOVA) with a two-tailed Student's *t*-test if the values followed a Gaussian distribution. If a normality test was not passed, or too few samples were present to estimate normality, a Kruskal–Wallis test, followed by a Mann–Whitney test for comparison between groups (indicated by an § where applicable), was used. All data are displayed as mean ± 1 SD. The level of statistical significance was set at *P* < 0.05.

## RESULTS

### Ex vivo reconditioning

During RC, the hearts resumed sinus rhythm within minutes of reperfusion. The mean average and mean maximum heart rate during RC were 149.81 ± 29.55 and 191.48 ± 47.96 bpm, respectively. Perfusate levels of lactate dehydrogenase, CK and troponin (378.89 ± 190 U/l, 59.22 ± 21.76 U/l and 13.46 ± 4.70 μg/l, respectively) were elevated compared with *in vivo* reference values, but were significantly lower than those after Langendorff reperfusion in all groups.

### Heart rate and contractility

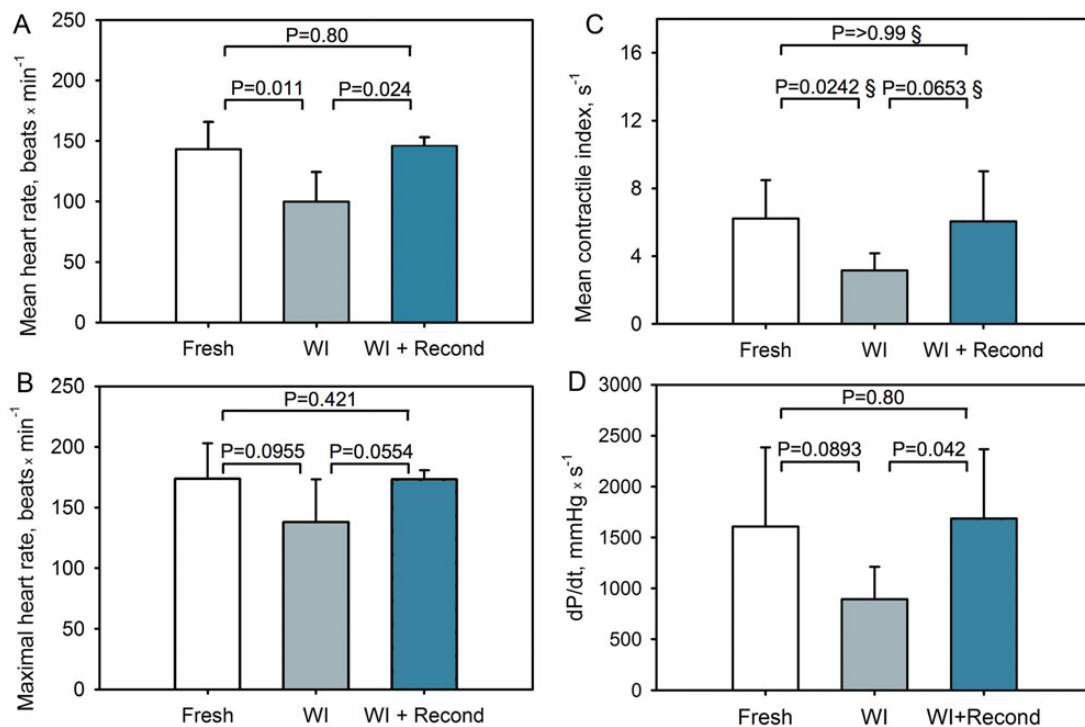
The mean and maximum heart rates of the reconditioned ischaemic hearts were higher than those of the ischaemic controls (145.80 ± 7.31 vs 99.76 ± 24.48 bpm *P* = 0.0024 respectively 173.18 ± 7.55 vs 137.94 ± 35.27 bpm *P* = 0.0554), and not significantly different from those of cold-stored fresh hearts (Fig. 2A and B). The reconditioned ischaemic hearts had a higher mean dP/dT (1687.14 ± 678.39 vs 1288.42 ± 434.48 mmHg/s *P* = 0.042) and mean contractile index (6.4 ± 2.88 vs 3.6 ± 0.548/s *P* = 0.006535) than non-treated ischaemic hearts, yet the difference was not statistically significant. No difference existed between reconditioned ischaemic hearts and cold-stored fresh controls (Fig. 2C and D).

### Myocardial damage

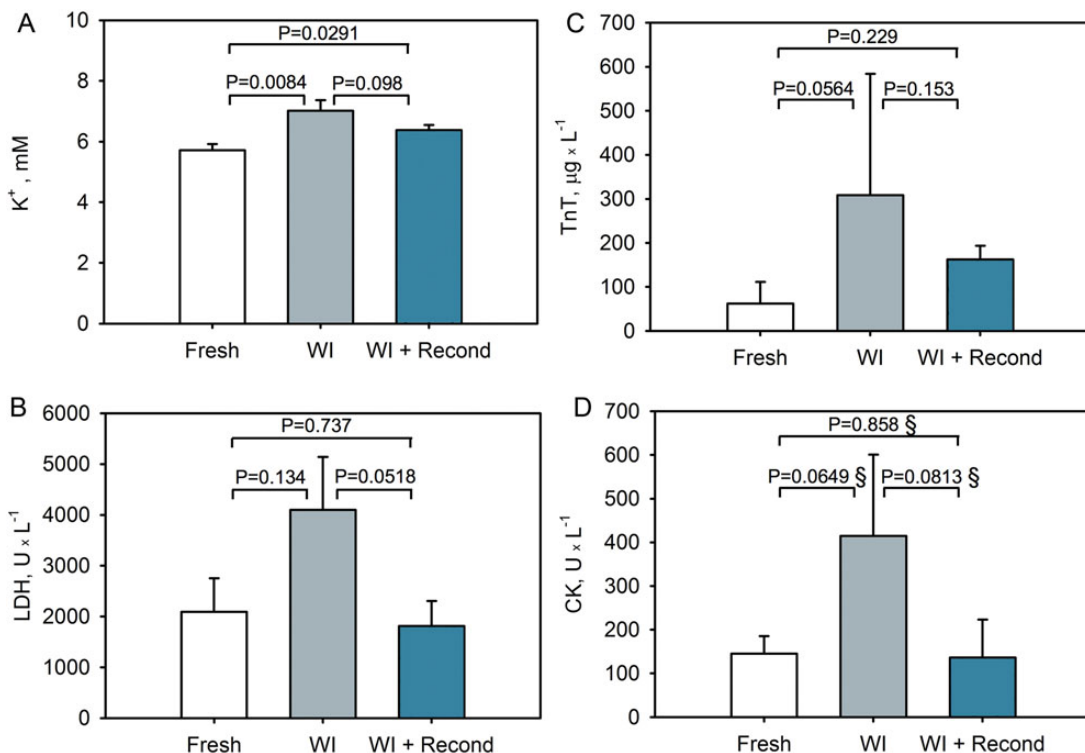
During Langendorff reperfusion of the ischaemic controls, perfusate levels of K<sup>+</sup> were significantly higher than those of fresh hearts (5.71 ± 0.20 vs 7.02 ± 0.35 mmol/l *P* = 0.0084; Fig. 3A). Perfusate levels of lactate dehydrogenase, CK and troponin T reconditioned ischaemic hearts were not significantly different from those of cold-stored ischaemic controls (Fig. 3B–D).

### Tissue energy state

Fresh-excised hearts had a tissue ATP content of 12.11 ± 2.07 μmol/g. Myocardial ATP dropped almost 10-fold to 1.40 ± 0.93 μmol/g after 25 min of WI. One hour of RC replenished ATP levels



**Figure 2:** The mean (A) and maximum heart rates (B), mean contractile index (C) and dP/dT (D) of fresh, ischaemic and reconditioned ischaemic hearts during Langendorff reperfusion.



**Figure 3:** Perfusate concentrations of K $^{+}$  (A), LDH (B), troponin T (C) and CK (D) of fresh, ischaemic and reconditioned ischaemic hearts during Langendorff reperfusion. LDH: lactate dehydrogenase; CK: creatine kinase.

to  $8.01 \pm 2.10$   $\mu$ mol/g (Fig. 4). During 4 h of CS, the ATP level of fresh and reconditioned ischaemic hearts dropped at a similar rate ( $1.56$  vs  $1.61$   $\mu$ mol/g/h) to  $5.87 \pm 1.65$  and  $1.75 \pm 0.48$   $\mu$ mol/g, respectively. Both values were significantly higher than those of ischaemic hearts ( $0.53 \pm 0.10$   $\mu$ mol/g  $P = 0.0159$  respectively

$P = 0.0159$ ). After Langendorff reperfusion, tissue ATP of both cold-stored fresh and reconditioned hearts was significantly higher than that of untreated cold-stored ischaemic controls ( $8.11 \pm 2.19$   $P = 0.0004$  respectively  $5.75 \pm 1.97$   $P = 0.031$  vs  $3.93 \pm 0.95$   $\mu$ mol/g; Fig. 4).

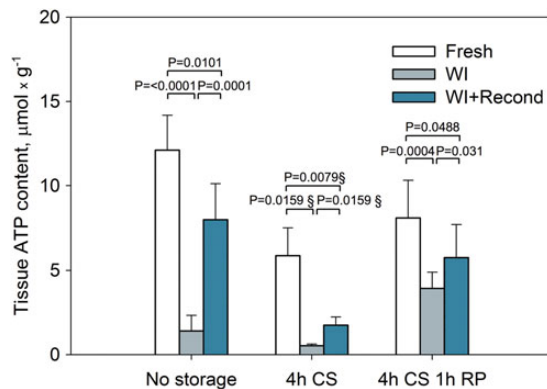


## Oxidative stress

After CS and following Langendorff reperfusion, the reconditioned ischaemic heart's tissue GSH content was  $1152.133 \pm 103.55$  respectively  $686.65 \pm 141.62$  nmol/g, compared with  $950.08 \pm 103.55$  respectively  $634.96 \pm 103.47$  nmol/g in the ischaemic control group ( $P=0.377$  respectively  $P=0.401$ ). The tissue redox state of the reconditioned ischaemic hearts, expressed as half-cell redox potential for the GSH-GSSG couple, was  $-264.43 \pm 8.05$  respectively  $-252.26 \pm 3.33$  mV at these time points. These values did not differ significantly from the values in the ischaemic control group ( $-263.10 \pm 5.83$  respectively  $-251.58 \pm 3.10$  mV  $P=0.791$  respectively  $P=0.672$ ).

## Histology

Compared with cold-stored fresh controls, the reconditioned ischaemic hearts showed minimal focal necrosis and oedema and

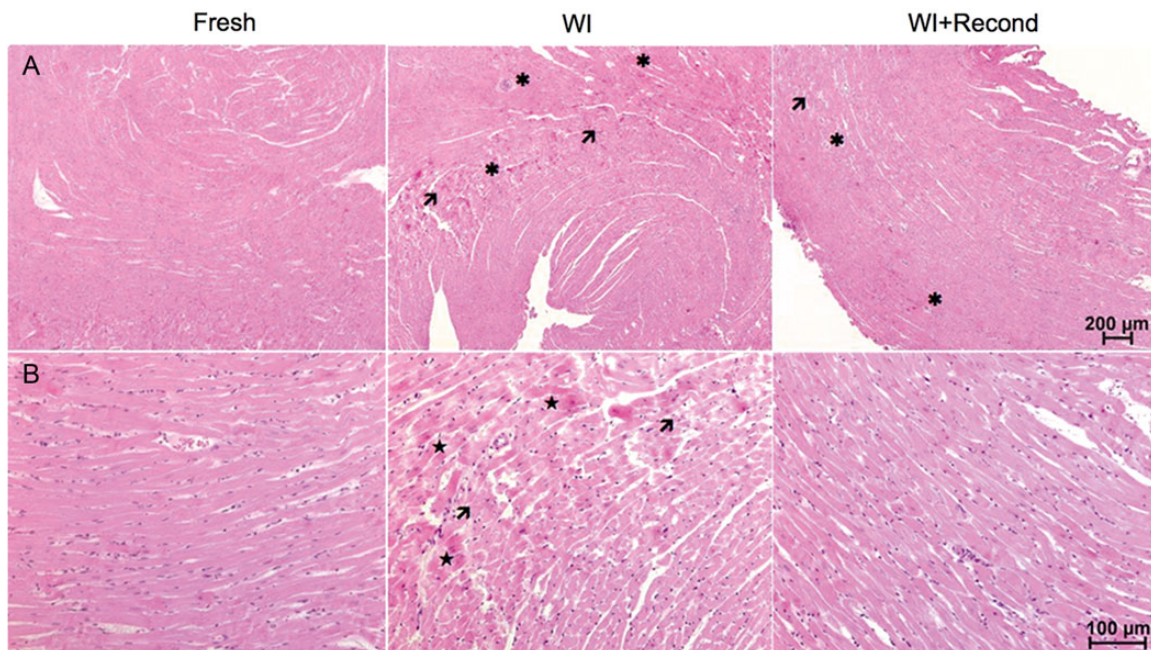


**Figure 4:** Myocardial ATP content of fresh, ischaemic and reconditioned ischaemic hearts before cold storage, after 4 h of cold storage and after Langendorff perfusion. ATP: adenosine triphosphate.

cold-stored ischaemic hearts showed widespread damage, oedema and extensive areas of infarction (Fig. 5). Fewer terminal deoxynucleotide transferase-mediated dUTP nick-end labelling-positive nuclei per slide were visible in reconditioned ischaemic hearts compared with cold-stored ischaemic hearts ( $73.66 \pm 36.53$  vs  $134 \pm 23.49$  nuclei/slide  $P=0.0341$ ), but their number was increased compared with cold-stored fresh controls ( $39.6 \pm 12.32$   $P=0.0074$ ).

## DISCUSSION

Machine perfusion (MP) has been investigated as a method for donor heart preservation since cardiac transplantation's early years. With the advent of advanced organ preservation solutions that permitted extended CS without compromising graft viability, MP was soon abandoned due to its complexity. Driven by the need for a preservation method that allows recovery and safe transplantation of hearts from DCD donors, MP has gained renewed interest. Two main MP approaches can be distinguished: hypothermic machine perfusion and NMP. During hypothermic machine perfusion, the heart is perfused with an oxygenated cardioplegic solution at  $0-4^{\circ}\text{C}$ . Hypothermic machine perfusion relies on the same principle as CS: cardioplegic arrest and cooling in order to minimize myocardial oxygen demand during preservation [9, 10]. During NMP, on the other hand, the heart is kept in a beating state by continuous perfusion with warm, oxygenated perfusion medium. Compared with CS, both hypothermic machine perfusion and NMP have enabled extended preservation and improved organ viability. However, because NMP's near physiological conditions allow temperature-dependent adaptive and reparative processes to take place, it is an ideal approach to recover organs after DCD. Nevertheless, compared with both CS and hypothermic machine perfusion, NMP is much more challenging to implement; a device capable of maintaining the heart in a beating state is likely to be difficult to transport and may require



**Figure 5:** Representative haematoxylin-eosin stained slides of fresh, ischaemic and reconditioned ischaemic hearts after Langendorff perfusion at  $5\times$  (A) and  $20\times$  (B) magnification. Arrows indicate tissue destruction/necrosis, asterisks indicate infarctions and stars indicate contraction bands.

constant monitoring, and malfunction during transportation could easily lead to loss of the graft.

To avoid these issues, we have assessed whether a short period of NMP directly following exposure to WI could reverse WI's harmful effects, permitting ensuing CS without jeopardizing graft viability. Following NMP, the reconditioned ischaemic hearts were stored for 4 h in cold preservation solution, after which graft performance was assessed in a blood-perfused Langendorff circuit and compared with that of cold-stored fresh and ischaemic controls.

Our results indicate that 1 h of *ex vivo* NMP enabled recovery of the heart's function and metabolic state to near-normal levels. During NMP, the hearts rapidly resumed sinus rhythm after a brief period of bradyarrhythmia. An initial drop in perfusate pH and rise in perfusate lactate levels were seen, and although the perfusate levels of lactate dehydrogenase, CK and troponin T were elevated compared with *in vivo* reference values, they were markedly lower than the same values of fresh cold-stored hearts during Langendorff reperfusion. This suggests that 4 h of CS by itself caused more myocardial damage than 25 min of WI, and that the combination of WI and CS is particularly harmful. During Langendorff reperfusion, both the heart rate and contractility of the reconditioned ischaemic hearts were higher than those of cold-stored ischaemic hearts and no different from those of fresh-excised cold-stored controls. However, in all groups, the mean maximum heart rate was lower than typical *in vivo* values.

We found that 25 min of WI led to an almost 10-fold reduction in tissue ATP. Although the impact of global WI on myocardial ATP is debated [16], ATP depletion is generally accepted as an important contributor to ischaemia-reperfusion damage. During one hour of normothermic reperfusion, tissue ATP levels were replenished to approximately two-thirds of those measured in freshly excised hearts, sufficient etc. to prevent ATP depletion during ensuing CS. Interestingly, both fresh-excised hearts and reperfused ischaemic hearts showed a constant rate of ATP decline during CS. ATP replenishment and hence tolerance to additional ischaemia during preservation and reimplantation may be further improved by fortifying the perfusate with metabolic substrates such as Krebs cycle intermediates and branched chain amino acids [16–19], which have shown to stimulate ATP synthesis during MP [20].

Oxidative stress is also seen as an important mediator of the damage after exposure to WI [21, 22]. However, we found no significant differences in the levels of GSH and tissue redox potential between the experimental groups. Nevertheless, oxidative stress during reperfusion may be reduced by using leucocyte-depleted blood as perfusate, shown to improve organ viability during MP [23], as well as by continuous delivery of antioxidants and radical scavengers during RC.

Both reconditioned ischaemic hearts and cold-stored ischaemic controls showed signs of inhomogeneous tissue perfusion, infarction and oedema. Tissue perfusion during RC may be improved by an initial 'hot shot' of cardioplegia, aimed at reducing myocardial oxygen demand during early reperfusion when the heart's microcirculation may still be compromised by post-ischaemic stunning and vasoconstriction. Focal ischaemia due to thrombi formation may be prevented by administering thrombolytic compounds such as streptokinase or thrombokinase during RC. Both the increased hydrostatic pressure during MP and the use of diluted blood as medium may have contributed to the tissue oedema we observed in all groups after reperfusion. This may be prevented by addition of compounds that increase the perfusate's oncotic pressure, such as dextrans or albumin [24].

We chose 37°C for RC, because we hypothesized that mimicking physiological conditions would permit optimal recovery from ischaemia. However, whether normothermia is necessary and even desirable remains to be investigated. It is conceivable that the protective effect of mild hypothermia commonly used in cardiac surgery may also be of benefit to our approach.

## Limitations of our approach

Although our *in vivo* model of WI more closely mimics a clinical DCD setting than other models [25], an *ex vivo* WI model may provide better control of both the duration and temperature of WI, thereby improving reproducibility. Moreover, since the heart is unloaded during the Langendorff reperfusion, myocardial damage might be underestimated, and because only left ventricular function is assessed, WI's impact on right ventricular function remains unknown. Even though this study is a proof of principle of the potential benefit of NMP after global WI, further refinement and validation of this approach are needed before testing in a large animal model is sensible.

## CONCLUSION

Our findings indicate when 25 min of WI is immediately followed by 60 min of *ex vivo* reconditioning, WI's negative impact is significantly ameliorated, enabling subsequent CS without compromising organ viability. Replenishment of intracellular ATP to near-normal levels during reperfusion, preventing ATP depletion during the CS interval, appears to be an important contributor to NMP's beneficial effect. We envision that a similar approach may eventually enable safe transplantation of hearts from DCD donors.

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**Conflict of interest:** none declared.

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## APPENDIX. CONFERENCE DISCUSSION

**Dr M. Erasmus (Groningen, Netherlands):** It's really a great challenge to use these kind of donors to expand the donor pool, and many researchers are busy finding out what the best options are. You chose warm reperfusion at, I think, the right time, immediately after the ischaemia. Why did you choose this normothermic reperfusion and not, say, 34 or 30 degrees as many others do?

**Dr Tolboom:** Well, I have two answers to that question. I've occupied myself with normothermic and sub-normothermic reperfusion for donor liver procurement and recovery since 2004, so I'm intimately familiar with what's needed to do this, so I just did what comes best, what's familiar to me.

The second part of the answer is that currently I'm looking at the whole spectrum between 20 and 37 degrees, and my intuition and my preliminary results say that actually the optimum temperature is somewhere between room temperature and normothermic. It's looking like 25 degrees is actually a very good temperature. If I go lower than 20, results aren't good, and actually the results of 37 degrees don't look as good as the results when I go a bit lower, like 25 degrees.

**Dr Erasmus:** Yes. But then in this research it looked like the warm reperfusion was as good as the control group because all the results were comparable, and only the cold storage did the damage. Did you expect that the cold storage would have such an impact on the damage?

**Dr Tolboom:** No, I did not. And with the cold stored group you mean, the group that was my control?

**Dr Erasmus:** Yes.

**Dr Tolboom:** I did not, no. I hadn't expected it, which I pointed out in the paper, I didn't show the results in the slides due to time limitations. When comparing the myocardial damage markers, the soluble ones in the perfusate, between the fresh hearts during Langendorff and the ischaemic hearts during the first reconditioning phase, the damage was way lower in the latter group. So 25 min of warm ischaemia inflicted way less damage than four hours of cold storage on healthy hearts. I didn't expect that.

**Dr Erasmus:** Oh, it surprised you also?

**Dr Tolboom:** It's an interesting finding, yes.

**Dr Erasmus:** My last question relates to the composition you used. You used blood and then diluted it.

**Dr Tolboom:** Yes.

**Dr Erasmus:** As others showed, especially German investigators, it's critical to have no leukocytes in this first reperfusion, and you do not mention a word about leukocytes.

**Dr Tolboom:** Yes. I recently recommenced this research in this field, and the goal of these experiments was to establish baseline parameters, and I hadn't expected that it would work so well. And that's what I did in my previous career in research doing the liver reperfusion; I leukocyte-depleted them. So that is something I'm going to be looking at. This is a very bare bones basic approach. Krebs-Henseleit mixed with blood is probably one of the worst conceivable perfusion solutions which makes its beneficial effects even more impressive to me. But definitely, yes, leukocytes are probably something that you want to avoid in a clinical setting.